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Japanese β°-Thalassemia: Molecular Characterization of a Novel Insertion Causing a Stop Codon

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During a physical checkup, a 42-year-old Japanese man with liver dysfunction was diagnosed as having β-thalassemia. Using molecular biological techniques including PCR, we investigated the chemical basis of the hematological disorder. We found that a frameshift attributable to the insertion of a thymidine into or following the TTT sequence of codon 42 transformed codon 43 (GAG) into a stop codon (TGA). We believe that this mutation has not been previously reported. © 1996 Wiley-Liss, Inc.

Key words: β-thalassemia, β-globin, polymerase chain reaction, cloning, sequencing, gene analysis, heterozygote, mutation, nonsense codon

INTRODUCTION

β-Thalassemia is a congenital abnormality characterized by reduced or absent β-globin chain synthesis [1]. It is caused by mutations in the β-globin gene or gene cluster. At a physical checkup, a 42-year-old nonsmoking Japanese man with liver dysfunction was found to have an array of hemoglobin abnormalities, including a high level of hemoglobin (Hb) A_2—suggestive of β-thalassemia. We analyzed red cell α- and β-globin biosynthesis in vitro using ^{3}H-leucine incorporation and found that β-globin synthesis was depressed, confirming the diagnosis. Using the polymerase chain reaction (PCR) on genomic DNA isolated from the buffy coat, we investigated the chemical basis of the patient's thalassemia. We discovered a nonsense mutation at codon 43 attributable to the insertion of a thymidine into or immediately following codon 42. This mutation has not been previously reported.

MATERIALS AND METHODS

Hematological and biochemical analysis of the patient's peripheral blood was carried out by standard methods.

Hemoglobin Study

Abnormal hemoglobin was detected by isoelectric focusing (pH range: 6–8) and HbA_2 and HbF levels were determined by anion-exchange (DEAE-5PW, 7.5 × 75 mm; Tosoh Co., Ltd., Tokyo, Japan) high-performance liquid chromatography (HPLC) with the gradient buffer (9 mM Tris–HCl, pH 8.0, and 0.5 M NaCl–9 mM Tris–HCl, pH 8.0) used as the elution buffer.

Hemoglobin Biosynthesis and Analysis

About 0.2 ml of the upper phase of centrifuged red cells was incubated at 37°C for 2 hr in amino acid medium containing ^{3}H-Leu (100 μCi), and the cells were washed and lysed in 0.01% saponin. The globin prepared from the hemolysate was fractionated on a CM-cellulose column (CM-52, Whatman Paper Co., Kent, UK; column size, 0.7 × 10 cm; elution buffer, 8 M–urea–Na phosphate buffer, pH 6.75, Na^+ 7→35 mM). The ratio of β- to α-chain biosynthesis was assumed equivalent to the ratio of their radioactivity, which was determined with a liquid scintillation counter [2].

Isolation of Genomic DNA

Genomic DNA was prepared by the method of Poncz et al. [3] from the buffy coat collected from peripheral blood.

Isolation of mRNA and Preparation of cDNA

mRNA was isolated from the patient's peripheral blood using a QuickPrep™ Micro mRNA purification kit (Phar-

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TABLE I. Hematological Findings

Variable	Value	Normal range
WBC × 10^9/L	6.9	3.5–9.5
RBC × 10^{12}/L	6.45	4.10–5.40
Hb g/dl	12.8	13.5–16.5
PCV L/L	0.422	0.39–0.48
MCV fl	65.4	87.0–103.0
MCH pg	19.8	29.0–35.0
MCHC g/dl	30.3	33.0–36.0
Reticulocyte (%)	1.4	0.5–1.5
HbA_2 (%)	7.91	2.2–3.2
HbF (%)	3.11	<1.2
β/α	0.46	0.9–1.2

macia P-L Biochemicals, Uppsala, Sweden) and cDNA was prepared using a First-Strand cDNA synthesis kit (Pharmacia P-L Biochemicals), both according to the manufacturer's instructions.

PCR Amplification of Genomic DNA and cDNA and the Primer Sets

PCR amplification was done with a GeneAmp kit (Perkin-Elmer Cetus, Norwalk, CT). The PCR with genomic DNA was carried out separately for each part of the β-globin gene with the gene divided at the center of intervening sequence (IVS) 2 [2], and the primer sets used for amplification were the same. The PCR primers used for cDNA wre as follow: 5′-TTTGCATGCGCTTC-TGACACAACTGTGTTC-3′ (underlined sequence is the *Sph*I site) for the 5′-site of the β-globin gene and 5′-TTTAAGCTTGCAGAATCCAGATGCGACCGG-3′ (underlined sequence is the *Hin*dIII site) for the 3′-site of the β-globin gene. The PCR conditions were the same as those for genomic DNA, except that the annealing temperature was 60°C, whereas it was 55°C for genomic DNA.

Preparation of ssDNA

The amplified genomic DNA was digested with two sets of enzymes, *Sph*I and *Hin*dIII and *Hin*dIII and *Pst*I, and the amplified cDNA was digested with *Sph*I and *Hin*dIII. The DNA was then ligated into the bacteriophage vector M13mp18 or M13mp19 at the restriction sites [4]. The recombinant DNA was transferred into the bacterium JM109 using an *Escherichia coli* Pulser (Nippon BioRad Laboratories, Tokyo, Japan), poured into 1 ml SOB (Tryptone 20 g, yeast extract 5 g, NaCl 0.585 g, and KCl 0.185 g dissolved in H_2O to 1,000 ml and autoclaved) culture medium (containing 0.2 M Mg^{2+} and 0.2% glucose), and incubated at 37°C for 1 hr. An aliquot (100 μl) of this bacterial preparation was incubated at 37°C for 6 hr in 10 ml SOB medium containing 0.2M Mg^{2+}, and then mixed ssDNA was prepared by standard methods. Next, 20 μl of the bacterial preparation was spread on a YT

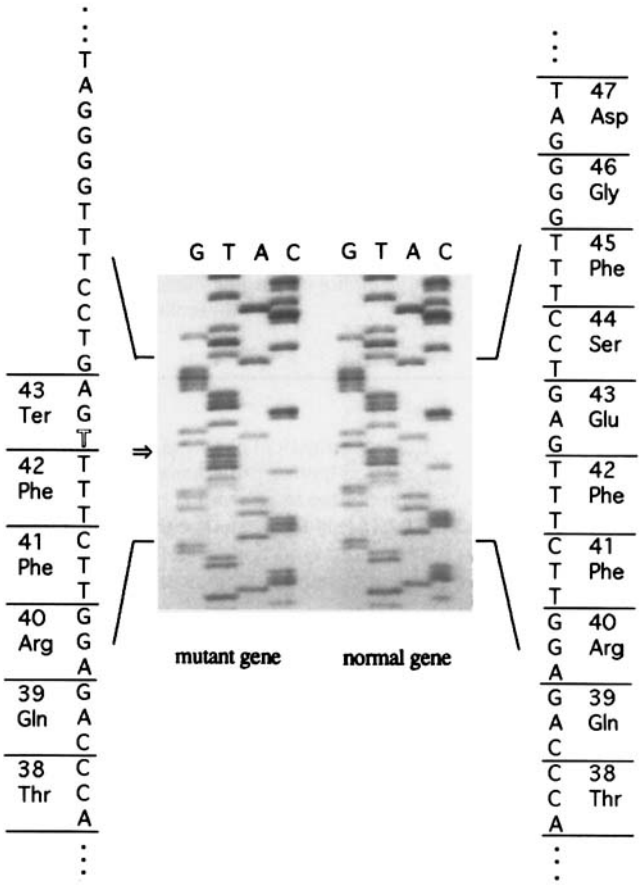

Fig. 1. Autoradiograms of the cloned ssDNA sequencing gel.

(tryptone 10 g, yeast extract 5 g, NaCl 5 g, and agar 15 g dissolved in 1,000 ml H_2O, autoclaved, and spread on an agar gel) plate containing IPTG (isopropyl-β-D-thiogalactopyranoside) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) to permit selection of the white plaques formed by recombinant DNA. The cloned ssDNA was prepared by the method described above [4].

Determination of Nucleotide Sequence

The nucleotide sequences were analyzed gradually from the 5′ to the 3′ end of the mixed and cloned ssDNAs by the dideoxy method using a DNA sequencing kit (Sequenase Version 2.0; U.S. Biochemicals, Cleveland, OH). Universal and synthetic nucleotides were used as sequencing primers.

RESULTS

Our hematological findings are shown in Table I. When the patient visited the hospital for a physical checkup, examination of his peripheral blood revealed microcytic hypochromic anemia and erythrocytosis. Mean corpuscular volume (MCV) and mean corpuscular hemoglobin

(MCH) were depressed. Isoelectric focusing revealed no abnormal hemoglobin, and HPLC revealed elevated levels of HbA_2 (7.91%) and HbF (3.11%). The peripheral blood smear showed many target cells and anisocytosis. In vitro analysis of globin biosynthesis showed the β/α globin chain ratio to be 0.46, indicating that β-globin chain synthesis was depressed. These findings are diagnostic for β-thalassemia.

An autoradiogram of mixed ssDNA prepared from genomic DNA showed no abnormal sequences in the promotor regions or splicing sites. The nucleotide sequence downstream of codon 43, however, was abnormal, and this was due to the insertion of a single thymidine into codon 42 or 43 (Fig. 1). This frameshift mutation changed the codon 42–43 sequence TTT-GAG to TTT-TGA; TGA is a termination codon. The polymorphisms at the third base of codon 2 (C/T) and IVS2-16 (G/C) of the mutant gene were C and G, respectively. The cloned ssDNA prepared from cDNA also contained the mutated sequence, indicating the presence of the abnormal mRNA in the reticulocytes.

DISCUSSION

The termination sequence in codon 43 of the patient's β-globin gene is a β°-thalassemia mutation and the chemical basis of the patient's hematological disorder. Mutant transcripts were found in the reticulocytes, suggesting that mutated polypeptides were synthesised in those cells. Such polypeptides would be truncated and nonfunctional.

Forty-four types of β-thalassemia following from frameshift mutations have been reported [5]. Fucharoen et al. [6], who investigated 11 different molecular defects in Japanese β-thalassemia (including some that have been found as well in non-Japanese populations), reported a novel β°-thalassemia frameshift mutation located at codon 15. Hattori et al. [7] reported that 6 of 17 families clinically diagnosed with β-thalassemia had frameshift mutations caused by a 4-nucleotide deletion (TTC-TTT→- - - -TT) at codons 41–42. Since codon 41–42 was involved in those mutations, and since codon 42–43 was involved in the new mutation reported here, codon 42 might be a "hot spot" for insertions and deletions.

Further studies of the families with these β°-thalassemia mutations are needed to clarify whether the mutations are inherited or de novo.

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